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FINAL REPORT

Study Title

Test for Chemical Induction of Chromosome Aberrations in Cultured Chinese Hamster Ovary (CHO) Cells With and Without Metabolic Activation

Test Article

3-Nitro-1,2,4-Triazol-5-one (NTO)

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Laboratory Project I.D.

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August 26, 2008

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October 30, 2008

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Aberdeen Proving Ground, MD

Sponsor's Study Coordinator

Gunda Reddy, Ph.D., DABT

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Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

3-Nitro-1,2,4-triazol-5-one (NTO, 99.6% pure) was tested for its potentials to induce chromosome aberrations in cultured Chinese Hamster Ovary (CHO) cells with and without metabolic activation according to OECD TG 473 in compliance with Good Laboratory Practice. NTO was found negative at concentrations of 5, 50, 100, 500, 1000, 2500, and 5000 ?g/mL in both with and without activation. A confirmatory chromosome aberration assay was performed without activation also showed negative.

15. SUBJECT TERMS

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STUDY DIRECTOR'S COMPLIANCE STATEMENT

Study No.: 0985-3110
The Sponsor's Test Article I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

The protocol (Appendix III) for this study was designed to meet or exceed the US EPA, OECD, and ICH Guidelines specified in the following documents:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Part 798, Health Effects Testing Guidelines, Subpart F Section 798.5375, *In Vitro* Mammalian Cytogenetics. Revised July 1, 2002.

OECD Guideline for the Testing of Chemicals, No. 473. *In Vitro* Mammalian Chromosome Aberration Test. Adopted July 21, 1997.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996.

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards with the exception that the dosing solution analysis was not conducted:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 2005.

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 2005.

Japanese Ministry of Agriculture, Forestry and Fisheries, 11 NohSan, Notification No. 6283, October 1, 1999.

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984.

Organization for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45 [ENV/MC/CHEM(98)17], Paris 1998.

Signature: _____ 10-30-08

Jian Song, Ph.D.
Study Director

Date

QUALITY ASSURANCE UNIT'S STATEMENT

Study No.: 0985-3110
Sponsor's Test Article I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

The performance of this study was audited for adherence to the Good Laboratory Practice regulations for nonclinical laboratory studies by the Quality Assurance Unit of SITEK Research Laboratories. In this context, the facilities, equipment, personnel, methods, practices, controls, original data and reports have been inspected as per SITEK's Quality Assurance Unit's Standard Operating Procedures. The information contained within this report accurately reflects the raw data generated from this study.

Protocol Review Date: 08-26-08

The following phases were inspected for this study:

Inspection Date	Phases Inspected	Date Findings Reported to Study Director	Date Findings Reported to Management
09-25-08	Weighing of the Test Article	10-06-08	10-17-08
10-15-08	Workbook Audit	10-15-08	10-16-08
10-15-08	Draft Report Audit	10-17-08	10-17-08
10-29-08	Final Report Audit	10-29-08	10-30-08

Signature Vian Lambert 10-30-08

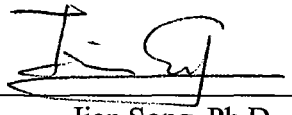
Vian Lambert, B.S.
Quality Assurance Manager

Date

STUDY DIRECTOR SIGNATURE PAGE

This study was performed under the supervision of Jian Song, Ph.D., Study Director, for in vitro cytogenetic assays at SITEK Research Laboratories, 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

The Final Report for this study was written by Dr. Song and released on October 30, 2008.

Signature:  10-30-08

Jian Song, Ph.D.
Study Director

Date

ABSTRACT

The results of Chromosome Aberration Assay suggest that the test article, 3-Nitro-1,2,4-Triazol-5-one (NTO, 99.6% pure), does not exhibit clastogenic potential.

The test article, NTO, was evaluated with and without exogenous metabolic activation for its potential to induce chromosome aberrations in cultured Chinese Hamster Ovary (CHO) cells. The test article was prepared and diluted with DMSO. In order to assess the toxicity of the test article, a Range Finding Test was performed. Based on the solubility test, the test article was evaluated at concentrations of 0.5, 1.0, 5.0, 10.0, 50.0, 100.0, 500.0, 1000.0 and 5000.0 µg/mL both with and without metabolic activation. DMSO and water were included in both systems as the solvent and untreated controls respectively.

In the non-activated system, duplicate cultures at each concentration level were treated for 3 hours in modified McCoy's 5A medium containing 10% fetal bovine serum. In the activated system, duplicate cultures at each concentration level were treated for 3 hours in serum-free medium containing phenobarbital/ß-naphthoflavone-induced rat liver S-9 fraction. The cells were harvested approximately 18 hours after the initiation of treatment (1.5 × normal cell cycle) in both systems, with 0.1 µg/mL Colcemid® present during the final 2 hours of incubation. Toxicity was determined by the reduction in relative cell growth (RCG) and/or relative mitotic index (RMI) in the treated cells, as compared to the cells treated with the solvent control. No significant cytotoxicity was observed at all dose levels both with and without metabolic activation.

Based on the results of the Range Finding Test, the Definitive Chromosome Aberration Assay was performed using test article concentrations of 5.0, 50.0, 100.0, 500.0, 1000.0 and 5000.0 µg/mL both without and with metabolic activation. Concurrent solvent and positive controls were also included. Duplicate cultures were treated at each concentration for 3 hours. The harvest time was 18 hours (1.5 × normal cell cycle) after the initiation of treatment in both systems with 0.1 µg/mL Colcemid® present during the final 2 hours. Mitomycin-C (MMC), at 0.4 and 0.8 µg/mL and Cyclophosphamide (CP), at 7.5 and 12.5 µg/mL, were used as the positive controls in the non-activated and activated systems, respectively.

Chromosome aberrations were scored from the cells treated with the concentrations of 500.0, 1000.0 and 5000.0 µg/mL both without and with activation. The untreated, the corresponding solvent control and one concentration each of the positive controls (MMC at 0.8 µg/mL and CP at 7.5 µg/mL) were also scored. Two hundred metaphases were scored from each concentration and the controls. Statistical analysis using the Chi-square test was performed. Toxicity was measured by determining the RCG and/or RMI. In addition, the percentage of polyploid and endoreduplicated cells were also determined at each concentration. Both the solvent control and positive controls in the Definitive Chromosome Aberration Assay fulfilled the requirements of a valid test. The results from the Definitive Assay were negative both with and without and activation.

A Confirmatory Chromosome Aberration Assay was performed without activation only, since the results from the Definitive Assay were negative in the non-activated system. The concentrations tested were 100.0, 500.0, 1000.0, 2500.0 and 5000.0 µg/mL. The treatment time was 18 hours. The harvest time was 18 hours after the initiation of treatment (1.5 × normal cell cycle). The untreated, solvent controls and positive controls (MMC at 0.2 and 0.4 µg/mL) were also included concurrently. Chromosome aberrations were scored from the cells treated at concentrations of 1000.0, 2500.0 and 5000.0 µg/mL, untreated, solvent and 0.4 µg/mL MMC. Both the solvent and positive controls in the Confirmatory Assay fulfilled the requirements of a valid test. The results from the Confirmatory Assay were negative without activation.

The results from the Definitive and Confirmatory Chromosome Aberration Assays indicate that the test article, NTO, did not induce a statistically significant increase in the percentage of cells with aberrations both with and without metabolic activation compared to the solvent controls, at the concentrations tested. Therefore, under the conditions of this test and according to the criteria set for evaluating the test results, NTO was negative both with and without metabolic activation in the CHO Chromosome Aberration Assay.

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INTRODUCTION

The experimental part of this study was conducted by Jian Song, Ph.D., Adrienne Parker, B.S., Shashi Sharma, B.S., Weiyu Xie, M.D. and Hussain S. Shaffi, B.S., from September 4, 2008 to Oct. 2, 2008 at SITEK Research Laboratories. The experimental procedures used to perform this study are described by H.J. Evans (4).

The *in vitro* chromosome aberration test is designed to identify agents that cause structural chromosome aberrations in cultured mammalian cells. Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumor suppressor genes of somatic cells are involved in cancer induction in human and experimental animals.

The purpose of this study was to evaluate the test article, 3-Nitro-1,2,4-Triazol-5-one (NTO), for its potential to induce genetic damage as manifested by the induction of chromosome aberrations in cultured Chinese hamster ovary (CHO) cells. CHO cells have been used extensively in the Chromosome Aberration Assay, and have been demonstrated to be effective in detecting the clastogenic activity of chemicals from a wide range of chemical classes (4-6).

MATERIALS

TEST ARTICLE

1. Name:	3-Nitro-1,2,4-Triazol-5-one (NTO)
2. CAS No.:	932-64-9
3. Provided by:	US Army Center for Health Promotion and Preventive Medicine Aberdeen Proving Ground, MD
4. Batch/Lot No.:	BAE 07B 305-001
5. Physical Description:	White Powder
6. Shipping Conditions:	Room Temperature
7. Date Received at SITEK:	July 23, 2008
8. Storage Conditions:	Refrigerated (1-5 °C)
9. Purity:	99.6%
10. Expiration Date:	Not Available

CONTROL ARTICLES

Positive Controls

Mitomycin-C (MMC), which induces chromosome aberrations in the absence of metabolic activation, was used at 0.4 and 0.8 µg/mL for Definitive Chromosome Aberration Assay and 0.2 and 0.4 µg/mL for Confirmatory Chromosome Aberration Assay, in the non-activated system. Information on the MMC used in this study is provided below:

1. Source:	Sigma Chemical Company
2. CAS No.:	50-07-7
3. Lot No.:	103K0499
4. Storage Conditions:	1-5°C
5. Expiration Date:	August 5, 2010

Cyclophosphamide (CP), which induces chromosome aberrations in the presence of metabolic activation, was used at 7.5 and 12.5 µg/mL for Definitive Chromosome Aberration Assay in the activated system. Information on the CP used in this study is provided below:

1. Source:	Sigma Chemical Company
2. CAS No.:	6055-19-2
3. Lot No.:	075K1661
4. Storage Conditions:	1-5°C
5. Expiration Date:	May 2, 2011

MMC and CP were dissolved in deionized, distilled water (ddH_2O) to prepare the stock solutions. MMC was diluted to 40 and 80 µg/mL and CP was diluted to 1.25 and 1.5 mg/mL. All stock solutions were dispensed in small aliquots and stored at -10 to -20°C. One vial of each was thawed just prior to treatment and used in treating the cells. For the Definitive Chromosome Aberration Assay, 50 µL of 40 µg/mL and 80 µg/mL MMC were added to 5 mL of medium to obtain the final concentrations of 0.4 and 0.8 µg/mL, respectively. 25 µL of 1.5 mg/mL and 50 µL of 1.25 mg/mL of CP stock solutions were added to 5 mL of medium to give final concentrations of 7.5 and 12.5 µg/mL, respectively. For the Confirmatory Chromosome Aberration assay, 25 µL and 50 µL of 40 µg/mL MMC was added to 5 mL of medium to obtain the final concentrations of 0.2 and 0.4 µg/mL, respectively.

The stability of MMC and CP, under the experimental conditions, was not determined by SITEK Research Laboratories. However, both substances were used before their expiration dates. Certificates of Analysis and their stability information can be obtained from Sigma – Aldrich.

Solvent Controls

The test article dosing solutions were prepared in DMSO. Therefore, DMSO was used as the solvent control for the test article. Information on the DMSO is provided below:

1. Source:	Sigma Chemical Company
2. Lot No.:	056K0172
3. Storage Conditions:	Room Temperature
4. Expiration Date:	January 16, 2012

INDICATOR CELLS

The clone CHO-W-B1 of the CHO cell line, used in this study, originated at Litton Bionetics and was obtained by SITEK through the Environmental Health Research and Testing Laboratories, Lexington, Kentucky, in 1988. The doubling time of this cell line is approximately 12 hours, and its modal chromosome number is 21. The karyotype analysis of the cell line is periodically performed and documented at SITEK Research Laboratories.

CULTURE MEDIUM

The McCoy's 5A medium used in this study was obtained from Gibco-BRL. Information on the medium used in this study is provided below:

1. Source:	Gibco BRL
2. Lot No.:	414032
3. Storage Conditions:	1-5°C
4. Expiration Date:	February, 2009

The fetal bovine serum was obtained from Gibco BRL. It was heat inactivated prior to use in this study. The antibiotics, (penicillin and streptomycin) and the supplement, (L-glutamine), used in this study were also obtained from Gibco-BRL. The lot numbers were recorded in the study workbook.

METABOLIC ACTIVATION SYSTEM

The metabolic activation mixture was prepared by SITEK Research Laboratories and it consisted of phenobarbital-5,6-Benzoflavone (phenobarbital/ß-naphthoflavone) -induced rat liver homogenate (S-9 fraction) and the cofactor pool (7). Prior to use in the assay, the S-9 was evaluated for its potential to induce an acceptable level of aberrations in Chinese hamster ovary cells with CP (7.5 µg/mL and 12.5 µg/mL). Immediately prior to use, the S-9 was thawed at room temperature and mixed with the cofactor pool to form the metabolic activation mixture, which consisted of 4mM NADP, 5mM glucose-6-phosphate, 30mM KCl, 10mM $MgCl_2$, 50mM sodium phosphate (pH 7.4) and 100 µL/mL of S-9 fraction (10%). This mixture was diluted 1:4 by volume with serum-free medium before treating the cultures.

The source, lot numbers, protein content, inducing agent, storage condition and expiration date are listed below:

1. Source:	Moltox
2. Lot No.:	2059
3. Protein Content:	32.1 mg/mL (Lot No.: 2059)
4. Inducing Agent:	Phenobarbital-5,6-Benzoflavone
5. Storage Condition:	≤ -70 °C
6. Expiration Date:	September 7, 2008 (Lot No.: 2059)*

Detailed information about the S-9 batch used in the Assay is provided in Appendix IV.

* The expiration date was extended one more month to October 7, 2008 after consulted to the vendor.

EXPERIMENTAL PROCEDURES

DOCUMENTATION

The materials, experimental procedures used in the performance of the study, experimental results and methods used in the evaluation of the results were documented in the study workbook.

TEST SYSTEM IDENTIFICATION

All of the test cultures were labeled using an indelible ink pen with the SITEK study number, control or test article concentration (use for treatment), the activation system followed by a code number (used for slide labeling) for the concentration tested, A or B to designate tubes receiving the same treatment (two replicate cultures) and the date of harvest. The slides were labeled with the SITEK study number, code number for the concentration tested, followed by A or B for two replicate cultures, and the date on which the slides were prepared.

SOLUBILITY TEST

The test article was tested for its solubility in H_2O and DMSO, in weight per volume. 25-100 mg of the test article was weighted and solvent in 0.1 mL increments was added, with thorough mixing between additions, until the test article is dissolved or until 1.5 mL of solvent has been added to the vessel. If the test article does not dissolve in 1.5 mL of solvent, more solvent will be added in aliquots of 0.5 mL until 5.0 mL has been added. The volume of solvent required for complete dissolution, and any additional observations, will be recorded in the study workbook. Test articles that do not dissolve in 5.0 mL of solvent will be recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

DETERMINATION OF pH

To determine the pH of the test article, 50 µL of test article at 500 mg/mL in DMSO was added to 5.0 mL of complete medium, resulting in a final test article concentration of 5000 µg/mL in medium. If the test article cause a change in the color of the medium, indicating a change in pH then the pH will be measured. It would be necessary to adjust the pH of the treatment medium prior to adding it to the cultures (8).

PREPARATION OF TEST CULTURES

Stock cultures, growing in T-75 cm^2 tissue culture flasks in antibiotic-free medium and showing 50-70% confluency, were harvested and used to prepare the test cultures. The culture

medium from the T-75 cm^2 flasks was discarded, and the cells were washed with Ca^{++}- and Mg^{++}-free phosphate buffered saline (PBS). Cells were then dissociated by incubation at 37 ± 1°C with 0.05% trypsin. The cells were resuspended in complete culture medium containing 10% HIFBS, 2 mM L-glutamine, 50 units/mL of penicillin and 50 µg/mL of streptomycin. The cell suspensions were pooled, and an aliquot of the cell suspension was diluted to the appropriate concentration and counted using a cell counter. Based on the cell counts, a separate cell suspension with $1x10^5$ cells/mL was prepared in complete medium. Five (5.0) mL of this suspension was seeded in each T-25 cm^2 tissue culture flask to give $5x10^5$ cells per flask. These cultures were used in the Range Finding Test and the Chromosome Aberration Assay. The culture flasks were incubated at 37°C, 5% CO_2 for approximately 20-24 hours prior to treatment.

PREPARATION OF METABOLIC ACTIVATION SYSTEM

The metabolic activation mixture consisted of phenobarbital/ß-naphthoflavone induced rat liver homogenate (S-9 fraction) and the cofactor pool. The S-9 fraction was stored at or below -70°C in small aliquots. The S-9 was validated for acceptable levels of protein content and metabolic activity. Immediately prior to use, the S-9 was thawed at room temperature and mixed with the cofactor pool to form the metabolic activation mixture which consisted of 4mM NADP, 5mM glucose-6-phosphate, 30mM KCl, 10mM $MgCl_2$, 50mM sodium phosphate (pH 7.4) and 100 µL/mL of S-9 fraction. This mixture was diluted 1:4 by volume with serum-free medium and used in refeeding the cultures.

PREPARATION OF TEST ARTICLE DOSING SOLUTIONS

The test article dosing solutions were prepared just prior to treatment. The test article was measured and an appropriate amount of DMSO was added to prepare the highest concentration for the Range Finding Test, Definitive or Confirmatory Assay. The remaining dosing solutions were prepared by subsequent dilutions.

RANGE FINDING TEST

In order to determine the toxicity of the test article, a Range Finding Test was performed.

Test cultures seeded approximately 24 hours earlier were used in the Range Finding Test. Two replicate cultures were used at each concentration level in both systems. Based on the solubility test, the Range Finding Test was conducted at concentrations ranged from 0.5 to 5000.0 µg/mL. The cytotoxicity of the test article was assessed by determining the Relative Cell Growth (RCG) and /or the Relative Mitotic Index (RMI) of the treated cells.

In the non-activated and activated systems, the culture medium was removed from the flasks and 5.0 mL of fresh, complete medium or 5.0 mL of serum-free medium with the S-9

activation mixture were added to each flask, respectively. The cells were exposed to the test article for 3 hours. The medium was then removed, and the cells were rinsed with DPBS, refed with 5.0 mL of complete medium and incubated for an additional 15 hours, with 0.1 µg/mL Colcemid present during the final 2 hours.

All of the cultures were harvested 18 hours after the initiation of treatment (1.5 x normal cell cycle time). The medium with dividing cells was transferred into labeled centrifuge tubes, and the monolayer of cells was washed with PBS, dissociated with 0.05% trypsin and resuspended in the collected medium. An aliquot of this cell suspension was counted using an electronic cell counter. The number of cells per flask was calculated for each concentration, and the Relative Cell Growth (RCG) was calculated using an Excel 2003 spreadsheet program with the following formula:

$$RCG = \frac{\text{No. Cells in Test Flask}}{\text{No. Cells in Solvent Flask}} \times 100$$

The remaining cell suspension was processed to determine the Relative Mitotic Index (RMI) as described below.

The cells were collected by centrifugation (800 rpm), swelled in hypotonic KCl (0.075M) and fixed in methanol: glacial acetic acid (3:1) fixative. The fixed cells were stored at 1-5°C. The cells were then collected again by centrifugation, resuspended in a small volume of fresh fixative and dropped on microslides. The slides were air dried, stained in 5% Giemsa stain and mounted in Cytoseal using #1 cover glasses. The coded slides were scored for Mitotic Index (MI). A total of 1000 cells were scored from each concentration (500 from each duplicate flask), and the number of dividing cells were recorded. The MI for each concentration was calculated using an Excel 2003 spreadsheet program with the following formula:

$$MI = \frac{\text{No. of Dividing Cells from 1000 Cells}}{10}$$

$$RMI = \frac{\text{Test Concentration MI}}{\text{Solvent Control MI}} \times 100$$

The cytotoxicity was evaluated on the basis of the reduction in the RCG and/or RMI. If possible, a concentration causing approximately 50% reduction in RCG and/or RMI was selected as the highest test concentration for the Chromosome Aberration Assay. In addition, three or more lower concentrations were included in the Assay. If no cytotoxicity was observed at the maximum concentration tested, the Chromosome Aberration Assay was performed at four decreasing concentrations starting with the maximum soluble concentration or one or two concentrations with precipitate.

DEFINITIVE CHROMOSOME ABERRATION ASSAY

Based on the results of the Range Finding Test, the Definitive Chromosome Aberration Assay was performed. The Definitive Chromosome Aberration Assay was conducted with a single harvest at 1.5 x normal cell cycle time.

The test cultures were prepared as described earlier. Two replicate cultures, seeded with 1x 10^5 cells/mL each approximately 20-24 hours earlier, were treated at each concentration level in the non-activated and activated systems. The cells were treated at concentrations of 5.0, 50.0, 100.0, 500.0, 1000.0 and 5000.0 µg/mL both with and without metabolic activation. Mitomycin-C (MMC), at 0.4 and 0.8 µg/mL and Cyclophosphamide (CP), at 7.5 and 12.5 µg/mL, were used as the positive controls in the non-activated and activated systems, respectively.

In the non-activated and activated systems, the culture medium was removed from the flasks and 5.0 mL of fresh, complete medium or 5.0 mL of serum-free medium with the S-9 activation mixture was added to each flask, respectively. The cells were exposed to the test article for 3 hours. The medium was then removed, and the cells were rinsed with DPBS, refed with 5.0 mL of complete medium, incubated for an additional 15 hours, with 0.1 µg/mL Colcemid present during the final two hours, and harvested 18 hours after the initiation of the treatment (1.5 x normal cell cycle time).

The cells were processed to determine the RCG and RMI as described in the Range Finding Test.

Parallel toxicity was assessed by a reduction in the RCG and/or RMI. The slides for the RMI were also used for the determination of chromosome aberrations.

Based on the RCG and/or RMI results, chromosome aberrations were scored from the cells treated with the concentrations of 500.0, 1000.0 and 5000.0 µg/mL both without and with activation. The untreated, corresponding solvent control and one concentration each of the positive controls (MMC at 0.8 µg/mL and CP at 7.5 µg/mL) were also scored. Two hundred metaphases were scored from each concentration and the controls. Statistical analysis was performed using the Chi-square test. Toxicity was measured by determining the RCG and RMI. In addition, the percentage of polyploid and endoreduplicated cells was also determined at each concentration.

The types of chromosome aberrations scored and the corresponding abbreviations used are given below (9):

1. Chromatid-type Aberrations

   Simple:

   tg - Chromatid gap - an achromatic region occurring along the length of a chromatid in which there is no misalignment.

tb - Chromatid break - a discontinuity occurring along the length of either of the two chromatids in which there is a misalignment.

isb - Isochromatid break - a discontinuity occurring in both the chromatids at the same locus showing complete rejoining or sister chromatid union at both the broken ends or incomplete rejoining, i.e., only at one of the two broken ends.

Complex:

qr - Quadriradial - chromatid interchanges between chromosomes leading to four-armed configurations. This could be asymmetrical with formation of a dicentric and an acentric chromatid, if union is complete, or symmetrical where there is no formation of a dicentric and an acentric chromatid.

tr - Triradial - isochromatid-chromatid exchanges resulting in three-armed configurations and sometimes fragments. The latter should not be scored as an independent aberration. The triradial could be monocentric or dicentric.

id - Interstitial deletion - intra-arm intra-changes resulting in deletion of small fragments which, however, stay in association with the parent chromatid.

ci - Chromatid intrachange - exchanges occurring between arms of the same chromosome resulting in asymmetrical (rings) or symmetrical configurations.

cr - Complex interchanges - multiarmed configurations resulting from breakage and reunion of two or more chromosomes.

2. Chromosome-type Aberrations

Simple:

sg - Chromosome gap - an achromatic region occurring in both chromatids of the chromosome at the same locus with no misalignment.

sb - Chromosome break - a discontinuity at the same locus in both chromatids, giving one acentric fragment which may be misaligned and a shortened monocentric chromosome, and where there is no sister chromatid union.

Complex:

d - Dicentric - an asymmetrical exchange between two chromosomes resulting in a chromosome with two centromeres with or without an accompanying acentric fragment which should not be score as a second aberration.

r - Ring - inter-arm intrachange happening within the chromosome, leading to formation of a centric ring with or without a chromosome fragment. The fragment should not be scored as a second aberration.

dm - Double minutes - intra-arm intrachanges leading to tight acentric paired rings.

3. Other Aberrations

pu - Pulverized chromosome or chromosomes - shattering of chromatid material resulting in several minute pieces. The identity of the chromosome is not decipherable. Considered as a single aberration.

sd - Severely damaged cell - cell with 10 or more aberrations.

pp - Polyploid cells - metaphases with multiples or approximate multiples of the haploid set of chromosomes. Not scored for structural aberrations.

e - Endoreduplication - metaphases with paired duplicated chromosomes or diplochromosomes; they are not scored for structural aberrations.

The chromosome aberration data from the score sheets were consolidated on a Summary Table using an Excel 2003 spreadsheet program. The number of aberrations per cell and the percentage of cells with one or more aberrations for each concentration level were calculated. The data were consolidated separately for the two cultures at each concentration, then pooled and presented together. Chromatid gaps and chromosome gaps were scored, but they were not included in calculating the percentage of cells with aberrations and the number of aberrations per cell. Of the remaining aberrations, each aberration scored was counted as one, except a severely damaged cell (sd), which was considered equal to 10 aberrations in calculating the number of aberrations per cell. Endoreduplicated and polyploid cells were recorded separately in percentages.

CONFIRMATORY CHROMOSOME ABERRATION ASSAY

A Confirmatory Chromosome Aberration Assay was performed without activation only, since the results from the Definitive Assay were negative. The concentrations tested were 100.0, 500.0, 1000.0, 2500.0 and 5000.0 µg/mL. The treatment time was 18 hours. The harvest time was 18 hours after the initiation of treatment (1.5 x normal cell cycle). MMC, at 0.2 and 0.4 µg/mL, was the positive control. Chromosome aberrations from the Confirmatory Assay were scored at concentrations of 1000.0, 2500.0 and 5000.0 µg/mL. The untreated, solvent and positive (MMC at 0.4 µg/mL) controls were also scored. Two hundred metaphases were scored from each concentration and the controls.

STATISTICAL ANALYSIS

The data for the percentage of cells with aberrations for each concentration were compared to the solvent control values using the Chi-square test. Results were considered significant if $p \leq 0.05$. Statistical analysis was not performed if the test concentration value was equal to or less than the concurrent or historical solvent control value.

If a positive response was indicated by the Chi-square test, the Cochran-Armitage test (trend test) was performed for evidence of a concentration-related response (10). The trend test was considered positive if $p \leq 0.05$.

CRITERIA FOR A VALID ASSAY

1. In the solvent control, the percentage of cells with aberrations should not exceed 4%.

2. At least 25% of the cells scored in the positive control should show one or more chromosome aberrations.

3. At least one of the test concentrations scored should show approximately 50% reduction in the RCG and/or RMI. This requirement should not be applied to test articles where no apparent toxicity could be achieved at the maximum soluble concentration or the highest allowable concentration.

EVALUATION OF TEST RESULTS

Positive Response

The test article was considered to have caused a positive response in this assay if the test article showed a positive concentration-response trend and a statistically significant increase over that of the solvent controls in the percentage of cells with aberrations at one or more

concentrations.

Negative Response

The test article was considered to have caused a negative response if none of the test concentrations showed a statistically significant increase in the percentage of aberrant cells.

Equivocal Response

The test article was considered to have caused an equivocal response if there was a statistically significant increase in the percentage of cells with aberrations without an accompanying positive concentration-response trend.

ARCHIVES

The raw data, documentation, protocol, protocol amendment/deviation and a copy of the Final Report, along with an electronic file containing data tables and the Final Report of the study, will be maintained for ten years at SITEK Research Laboratories archives at 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

RESULTS

SOLUBILITY TEST

A solubility test was performed. 50 mg of the test article was soluble with 3500 µl of water resulting in a final concentration of 14.28 mg/mL. 50 mg of the test article was soluble with 100 µl DMSO, resulting in a final concentration of 500 mg/mL. DMSO was selected as the solvent. 50 µL of the test article at 500 mg/mL in DMSO was added to 5.0 mL of complete medium resulting in a final test article concentration of 5000 µg/mL and formed a clear solution.

DERTERMINATION OF pH

50 µL of the test article at 500 mg/mL in DMSO was added to 5.0 mL of complete medium, resulting in a final test article concentration of 5000 µg/mL in medium and the color of the medium was changed pH = 3.06. Therefore 1 N HCL was added to adjust the pH of the treatment medium.

RANGE FINDING TEST

The results of the Range Finding Test indicated: The RCGs ranged from 137-255% in the non-activated system and 135-204% in the activated system, at concentrations of 0.5-5000.0 µg/mL (Appendix I, Table 1). The RMIs were from 55% - 114% at the concentration of 0.5-5000.0 µg/mL without activation. With activation, the RMIs were from 61 - 109% at the concentration of 0.5-5000.0 µg/mL (Appendix I, Table 2). No significant cytotoxicity was observed in the Range Finding Test.

DEFINITIVE CHROMOSOME ABERRATION ASSAY

Based on the toxicity results (RCGs and/or RMIs) from the Range Finding Test, the concentrations of 5, 100, 500, 1000, 5000µg/mL both without and with activation were tested.

The parallel toxicity results, as determined by the reduction in the RCG and/or RMI of the treated cells in the non-activated and activated systems, are presented in Tables 3 (RCG) and 4 (RMI) (Appendix I).

In the non-activated system, the RCGs for the test article concentrations of 5, 100, 500, 1000, 5000 µg/mL ranged from 95-139% and RMIs ranged from 68-107%. In the activated system, the RCGs for the test article concentrations of 5-5000 µg/mL ranged from 106-128% and RMIs ranged from 77-133%. Based on the toxicity results (RCGs and/or RMIs), the chromosome

aberrations were scored at the concentrations of 500, 1000 and 5000 µg/mL in both the non-activated and the activated systems. In addition, the corresponding untreated, solvent and positive (MMC at 0.8 µg/mL and CP at 7.5 µg/mL) controls were also scored. One hundred (100) metaphases were scored from each of the two replicate cultures for each concentration and the controls.

The results of the Definitive Chromosome Aberration Assay in the activated and non-activated systems are summarized and presented in Tables 5 and 6, respectively (Appendix I).

The averages of the percentage of cells with aberrations scored in the Definitive Chromosome Aberration Assay are summarized below:

Without Activation		With Activation	
Treatment (µg/mL) Treatment Time: 3 Hours	Chromosome Aberrations (% cells)	Treatment (µg/mL) Treatment Time: 3 Hours	Chromosome Aberrations (% cells)
Untreated (Water)*	0.0	Untreated (Water)*	0.5
Solvent (DMSO)*	1.0	Solvent (DMSO)*	1.0
NTO (500)	0.0	NTO (500)	0.0
NTO (1000)	0.5	NTO (1000)	0.0
NTO (5000)	0.0	NTO (5000)	0.0
MMC (0.80)	44.5**	CP (7.5)	34.5**

* Water was used as an Untreated Control and DMSO was used as Solvent Control.
** Statistically significant response using the Chi-square test ($P \leq 0.0001$)

CONFIRMATORY CHROMOSOME ABERRATION ASSAY

Only the non-activated system was tested in the Confirmatory Assay. The Confirmatory Assay was performed at the concentrations of 100, 500, 1000, 2500 and 5000 µg/mL. The parallel toxicity results of the Confirmatory Assay, as determined by the reduction in the RCG and/or RMI of the treated cells, are presented in Tables 7 (RCG) and 8 (RMI) (Appendix I).

The RCGs for the test article concentrations of 100-5000 µg/mL ranged from 63-131% and RMIs ranged from 35-88%. Based on the toxicity results (RCGs and/or RMIs), chromosome aberrations were scored from the concentrations of 1000, 2500 and 5000 µg/mL. In addition, the corresponding untreated, solvent and positive (MMC at 0.4 µg/mL) controls were also scored. One hundred (100) metaphases were scored from each of the two replicate cultures for each concentration and the controls.

The results of the Confirmatory Chromosome Aberration Assay are summarized and presented in Table 9 (Appendix I).

The averages of the percentage of cells with aberrations scored in the Confirmatory Chromosome Aberration Assay are summarized below:

Treatment (µg/mL) Treatment time: 18 hours	Average (% Cells with Aberrations) Without Activation
Untreated (Water)*	0.0
Solvent (DMSO)*	0.0
NTO (1000)	0.0
NTO (2500)	0.5
NTO (5000)	0.0
MMC (0.20)	40.5**

* Water was used as an Untreated Control and DMSO was used as Solvent Control.
** Statistically significant response using the Chi-square test ($P \leq 0.0001$).

STATISTICAL ANALYSIS

Statistical analysis indicated that the test article did not induce a statistically significant increase in the percentage of cells with aberrations over the solvent controls in the Definitive as well as the Confirmatory Chromosome Aberration Assays. Therefore, it was not necessary to perform a trend test.

The percentage of polyploidy (pp) was in the normal range (0-5.0%) in the Definitive Chromosome Aberration Assays with activation. Without activation, in both Definitive and the Confirmatory Chromosome Aberration Assays the percentage of polyploidy (pp) was higher than 5.0% in some groups but was not significantly changed as compared to solvent control. The percentage of endoreduplicated cells (e) ranged from 0.0-0.5% in both Definitive and Confirmatory Assays without activation which was in normal range (0-1.0%). With activation the percentage of endoreduplicated cells of 500 and 1000µg/mL treatment groups increased to 3% and when compared to solvent control may indicate that the test substance has the potential to inhibit cell cycle progression (11).

The SITEK's historical data for the negative controls (untreated and DMSO) are presented in Appendix II.

CONCLUSIONS

The results from the Definitive and Confirmatory Chromosome Aberration Assays indicate that the test article, 3-Nitro-1,2,4-Triazol-5-one (NTO, 99.6% pure) did not induce a statistically significant increase in the percentage of cells with aberrations both with and without metabolic activation when compared to the solvent controls, at the concentrations tested. Therefore, under the conditions of this test and according to the criteria set for evaluating the test results, NTO was negative both with and without metabolic activation in the CHO Chromosome Aberration Assay.

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APPENDIX I

DATA TABLES

TABLE 1
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
RCG - RANGE FINDING TEST

TEST ARTICLE: NTO
SPONSOR: US ARMY CHPPM
SOLVENT: DMSO
SITEK STUDY NO.: 0985-3110
TRIAL NO.: A1

WITHOUT ACTIVATION					WITH ACTIVATION				
Test Article Conc. (µg/mL)		No. of Cells per Flask	Mean No. of Cells X 10^6	RCG*	Test Article Conc. (µg/mL)		No. of Cells per Flask	Mean No. of Cells X 10^6	RCG*
Untreated	A	1.43			Untreated	A	1.00		
Untreated	B	1.61	1.52	208%	Untreated	B	1.10	1.05	202%
Solvent	A	0.65			Solvent	A	0.46		
Solvent	B	0.80	0.73	100%	Solvent	B	0.57	0.52	100%
0.5	A	1.68			0.5	A	0.78		
0.5	B	1.36	1.52	208%	0.5	B	0.62	0.70	135%
1	A	1.60			1	A	0.81		
1	B	1.75	1.68	230%	1	B	0.82	0.82	158%
5	A	1.94			5	A	0.69		
5	B	1.78	1.86	255%	5	B	0.77	0.73	140%
10	A	1.47			10	A	0.76		
10	B	1.50	1.49	204%	10	B	0.71	0.74	142%
50	A	0.91			50	A	0.90		
50	B	1.09	1.00	137%	50	B	1.09	1.00	192%
100	A	1.02			100	A	1.08		
100	B	0.97	1.00	137%	100	B	1.04	1.06	204%
500	A	1.28			500	A	0.92		
500	B	1.52	1.40	192%	500	B	0.90	0.91	175%
1000	A	1.50			1000	A	0.89		
1000	B	1.25	1.38	189%	1000	B	0.89	0.89	171%
5000	A	1.07			5000	A	0.82		
5000	B	1.32	1.20	164%	5000	B	0.73	0.78	150%

*RCG = Relative Cell Growth = $\frac{\text{No. of Cells in the Test Flask}}{\text{No. of Cells in the Solvent Flask}}$ X 100

Verified by: QA VL SD JS

Table 2

CHROMOSOME ABERRATION ASSAY IN CHO CELLS

MITOTIC INDEX - RANGE FINDING TEST

SPONSOR : US ARMY CHPPM — STUDY NO. : 0985 -3110

TEST ARTICLE : NTO — SOLVENT : DMSO — TRIAL NO.: A1

Without Activation - Treatment: 3 Hours Harvest: 18 Hours						With Activation - Treatment: 3 Hours Harvest: 18 Hours					
Test Article Concentration (µg/mL)	Tube No.		No. of Dividing Cells/500	Mean Mitotic Index	Relative Mitotic Index	Test Article Concentration (µg/mL)	Tube No.		No. of Dividing Cells/500	Mean Mitotic Index	Relative Mitotic Index
Untreated	**74**	A	122	22.1	78%	Untreated	**22**	A	47	8.3	56%
		B	99					B	36		
Solvent	**84**	A	171	28.4	100%	Solvent	**53**	A	86	14.9	100%
		B	113					B	63		
0.5	**96**	A	59	15.5	55%	0.5	**76**	A	82	14.9	100%
		B	96					B	67		
1	**94**	A	75	16.3	57%	1	**86**	A	43	10.9	73%
		B	88					B	66		
5	**3**	A	84	16.5	58%	5	**7**	A	51	9.1	61%
		B	81					B	40		
10	**75**	A	108	21.2	75%	10	**99**	A	44	12.0	81%
		B	104					B	76		
50	**32**	A	140	23.9	84%	50	**17**	A	66	12.3	83%
		B	99					B	57		
100	**57**	A	155	32.4	114%	100	**89**	A	50	9.9	66%
		B	169					B	49		
500	**45**	A	138	25.3	89%	500	**59**	A	67	13.9	93%
		B	115					B	72		
1000	**40**	A	107	22.9	81%	1000	**33**	A	64	13.1	88%
		B	122					B	67		
5000	**26**	A	162	29.3	103%	5000	**73**	A	69	16.2	109%
		B	131					B	93		

All test article concentrations were compared to solvent.

MI = No. of dividing cells scored from 1000 cells / 10

RMI = Test Dose MI X 100 / Solvent Control MI

Verified by: QA VL SD JS

TABLE 3
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
RCG - DEFINITIVE ASSAY

TEST ARTICLE: NTO
SPONSOR: US ARMY CHPPM
SOLVENT: DMSO
SITEK STUDY NO.: 0985-3110
TRIAL NO.: B1

WITHOUT ACTIVATION					WITH ACTIVATION				
Test Article Conc. (µg/mL)		No. of Cells per Flask	Mean No. of Cells X 10^6	RCG*	Test Article Conc. (µg/mL)		No. of Cells per Flask	Mean No. of Cells X 10^6	RCG*
Untreated	A	1.62			Untreated	A	1.16		
Untreated	B	1.44	1.53	103%	Untreated	B	1.08	1.12	90%
Solvent	A	1.47			Solvent	A	1.11		
Solvent	B	1.50	1.49	100%	Solvent	B	1.37	1.24	100%
5	A	1.39			5	A	1.54		
5	B	2.16	1.78	119%	5	B	1.58	1.56	126%
100	A	2.00			100	A	1.51		
100	B	2.14	2.07	139%	100	B	1.54	1.53	123%
500	A	1.74			500	A	1.60		
500	B	1.63	1.69	113%	500	B	1.58	1.59	128%
1000	A	1.80			1000	A	1.17		
1000	B	2.01	1.91	128%	1000	B	1.44	1.31	106%
5000	A	1.52			5000	A	1.43		
5000	B	1.32	1.42	95%	5000	B	1.28	1.36	110%
CP 7.5	A	1.05			MMC 0.4	A	1.17		
CP 7.5	B	1.03	1.04	68%	MMC 0.4	B	1.19	1.18	105%
CP 12.5	A	0.81			MMC 0.8	A	0.94		
CP 12.5	B	0.87	0.84	55%	MMC 0.8	B	0.99	0.97	87%

*RCG = Relative Cell Growth = $\frac{\text{No. of Cells in the Test Flask}}{\text{No. of Cells in the Solvent Flask}}$ X 100

Verified by: QA VL SD JS

Table 4

CHROMOSOME ABERRATION ASSAY IN CHO CELLS

RELATIVE MITOTIC INDEX - DEFINITIVE ASSAY

SPONSOR : Army, CHPPM — STUDY NO. : 0985 -3110

TEST ARTICLE : NTO — SOLVENT : DMSO — TRIAL NO.: B1

Without Activation - Treatment: 3 Hours Harvest: 18 Hours						With Activation - Treatment: 3 Hours Harvest: 18 Hours					
Test Article Concentration (µg/mL)	Tube No.		No. of Dividing Cells/500	Mean Mitotic Index	Relative Mitotic Index	Test Article Concentration (µg/mL)	Tube No.		No. of Dividing Cells/500	Mean Mitotic Index	Relative Mitotic Index
Untreated	**8**	A	81	16.5	83%	Untreated	**24**	A	145	27.7	133%
		B	84					B	132		
Solvent	**64**	A	97	19.8	100%	Solvent	**88**	A	91	20.8	100%
		B	101					B	117		
5	**30**	A	66	13.4	68%	5	**31**	A	111	22.6	109%
		B	68					B	115		
100	**16**	A	74	13.9	70%	100	**15**	A	145	27.6	133%
		B	65					B	131		
500	**72**	A	78	17.1	86%	500	**18**	A	122	23.8	114%
		B	93					B	116		
1000	**4**	A	86	17.4	88%	1000	**56**	A	132	24.7	119%
		B	88					B	115		
5000	**10**	A	104	21.1	107%	5000	**28**	A	87	16.0	77%
		B	107					B	73		
MMC 0.4	**12**	A	74	13.5	82%	CP 7.5	**95**	A	16	2.9	10%
		B	61					B	13		
MMC 0.8	**3**	A	32	6.0	36%	CP12.5	**7**	A	9	1.7	6%
		B	28					B	8		

All test article concentrations were compared to solvent.

Positive Controls were compared to Untreated since the Solvent for MMC and CP was water

$$MI = \frac{\text{No. of dividing cells scored from 1000 cells}}{10}$$

$$RMI = \frac{\text{Test Dose MI}}{\text{Solvent Control MI}} \times 100$$

Verified by: QA VL

SD JS

TABLE 5
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
CHROMOSOME ABERRATIONS - DEFINITIVE ASSAY

TEST ARTICLE: NTO
SPONSOR: Army CHPPM
SOLVENT: DMSO

TREATMENT TIME: 3 Hours
HARVEST TIME: 18 Hours

SITEK STUDY NO.: 0985-3110
TRIAL NO.: B1
METABOLIC ACTIVATION: Yes (X) No ( )

TREATMENT AND CONC. (µg/mL)	CELLS Scored	NUMBER AND TYPE OF ABERRATIONS																	NO. OF ABS. PER CELL	% CELLS WITH ABS.	P-VALUE IN CHI-SQUARE**
		NOT COMPUTED				Chromatid Type							Chromosme Type				Others				
						Simple		Complex					Simple	Complex							
		tg	sg	% pp	% e	tb	isb	tr	qr	cr	id	ci	sb	d	r	dm	pu	sd*			
Untreated A	100			0	0														0.00	0.0	
Untreated B	100			2	3	1													0.01	1.0	
Untreated A+B	200	0	0	1.0	1.5	1	0	0	0	0	0	0	0	0	0	0	0	0	0.005	0.5	
Solvent A	100			0	0	1	1												0.02	2.0	
Solvent B	100			3	0														0.00	0.0	
Solvent A+B	200	0	0	1.5	0.0	1	1	0	0	0	0	0	0	0	0	0	0	0	0.010	1.0	
500 A	100			0	2														0.00	0.0	
500 B	100			3	4														0.00	0.0	
500 A+B	200	0	0	1.5	3.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000	0.0	<Solvent
1000 A	100			1	3														0.00	0.0	
1000 B	100			5	3														0.00	0.0	
1000 A+B	200	0	0	3.0	3.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000	0.0	<Solvent
5000 A	100			0	1														0.00	0.0	
5000 B	100			3	0														0.00	0.0	
5000 A+B	200	0	0	1.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000	0.0	<Solvent
CP 7.5 A	100			0	0	11	9	6	11	5	4	1	5				3	2	0.75	41.0	
CP 7.5 B	100			11	0	4		7	7	2		2	4	6					0.32	28.0	
CP 7.5 A+B	200	0	0	5.5	0.0	15	9	13	18	7	4	3	9	6	0	0	3	2	0.535	34.5	P=0.0000

* sd = 10 aberrations in calculations.

In Chi-square test all test concentrations were compared to solvent, CP compared to untreated control data since solvent for CP was water.

Verified by: QA VL SD JS

TABLE 6
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
CHROMOSOME ABERRATIONS - DEFINITIVE ASSAY

TEST ARTICLE: NTO | TREATMENT TIME: 3 Hours | SITEK STUDY NO.: 0985-3110
SPONSOR: Army CHPPM | HARVEST TIME: 18 Hours | TRIAL NO.: B1
SOLVENT: DMSO | METABOLIC ACTIVATION: Yes ( ) No (X)

TREATMENT AND CONC. (µg/mL)	CELLS Scored	NUMBER AND TYPE OF ABERRATIONS																	NO. OF ABS. PER CELL	% CELLS WITH ABS.	P-VALUE IN CHI-SQUARE**
		NOT COMPUTED				Chromatid Type							Chromosme Type				Others				
						Simple		Complex					Simple	Complex							
		tg	sg	% pp	% e	tb	isb	tr	qr	cr	id	ci	sb	d	r	dm	pu	sd*			
Untreated A	100			0	0														0.00	0.0	
Untreated B	100			13	0														0.00	0.0	
Untreated A+B	200	0	0	6.5	0.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000	0.0	
Solvent A	100			1	0		1												0.01	1.0	
Solvent B	100			9	1	1													0.01	1.0	
Solvent A+B	200	0	0	5.0	0.5	1	1	0	0	0	0	0	0	0	0	0	0	0	0.010	1.0	
500 A	100			0	0														0.00	0.0	
500 B	100			10	0														0.00	0.0	
500 A+B	200	0	0	5.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000	0.0	<Solvent
1000 A	100			0	0	1													0.01	1.0	
1000 B	100			5	0														0.00	0.0	
1000 A+B	200	0	0	2.5	0.0	1	0	0	0	0	0	0	0	0	0	0	0	0	0.005	0.5	<Solvent
5000 A	100			0	0														0.00	0.0	
5000 B	100			11	0	1													0.01	0.0	
5000 A+B	200	0	0	5.5	0.0	1	0	0	0	0	0	0	0	0	0	0	0	0	0.005	0.0	<Solvent
MMC 0.8 A	100			0	0	8	7	10	9	11	3	6	9				4	3	0.97	47.0	
MMC 0.8 B	100			6	0	15		17	14		1	3	7		2				0.59	42.0	
MMC 0.8 A+B	200	0	0	3.0	0.0	23	7	27	23	11	4	9	16	0	2	0	4	3	0.780	44.5	P=0.000

* sd = 10 aberrations in calculations.

In Chi-square test all test concentrations were compared to solvent.
MMC compared to SITEK's historical Untreated control data (0.13%) since solvent for MMC was water and concurrent value is 0%.
Verified by: QA VL SD JS

TABLE 7
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
RCG - CONFIRMATORY ASSAY

TEST ARTICLE: NTO
SPONSOR: US ARMY CHPPM
SOLVENT: DMSO
SITEK STUDY NO.: 0985-3110
TRIAL NO.: B2

WITH ACTIVATION	WITHOUT ACTIVATION			
	Test Article Conc. (µg/mL)	No. of Cells per Flask	Mean No. of Cells X 10^6	RCG*
With Activation not Performed in B2	Untreated A Untreated B	1.53 1.46	1.50	104%
	Solvent A Solvent B	1.33 1.54	1.44	100%
	100 A 100 B	2.06 1.71	1.89	131%
	500 A 500 B	1.14 1.77	1.46	101%
	1000 A 1000 B	1.65 1.01	1.33	92%
	2500 A 2500 B	1.02 0.80	0.91	63%
	5000 A 5000 B	1.53 1.31	1.42	99%
	MMC 0.2 A MMC 0.2 B	1.47 1.43	1.45	97%
	MMC 0.4 A MMC 0.4 B	0.04 1.29	0.67	45%

*RCG = Relative Cell Growth = $\frac{\text{No. of Cells in the Test Flask}}{\text{No. of Cells in the Solvent Flask}}$ X 100

Verified by: QA VL SD JS

Table 8

CHROMOSOME ABERRATION ASSAY IN CHO CELLS

MITOTIC INDEX - CONFIRMATORY ASSAY

SPONSOR : US Army CHPPM STUDY NO. : 0985 -3110

TEST ARTICLE : NTO SOLVENT : DMSO TRIAL NO.: B2

Without Activation - Treatment:			18 Hours	Harvest: 18 Hours		With Activation - Treatment: Hours Harvest: Hours
Test Article Concentration (µg/mL)	Tube No.		No. of Dividing Cells/500	Mean Mitotic Index	Relative Mitotic Index	
Untreated	6	A	76	15.9	98%	Activated System was not performed in B2
		B	83			
Solvent	26	A	80	16.3	100%	
		B	83			
100	54	A	45	9.7	60%	
		B	52			
500	13	A	58	12.6	77%	
		B	68			
1000	65	A	60	11.8	72%	
		B	58			
2500	68	A	71	14.3	88%	
		B	72			
5000	52	A	25	5.7	35%	
		B	32			
MMC 0.2	61	A	36	7.9	50%	
		B	43			
MMC 0.4	57	A	20	4.7	30%	
		B	27			

All test article concentrations were compared to solvent.

Solvent for MMC is water so the positive control was compared to the untreated

MI = No. of dividing cells scored from 1000 cells / 10

RMI = Test Dose MI X 100 / Solvent Control MI

RMI = Positive Dose MI X 100 / Untreated Control MI

Verified by: QA VL SD JS

TABLE 9

CHROMOSOME ABERRATION ASSAY IN CHO CELLS

CHROMOSOME ABERRATIONS - CONFIRMATORY ASSAY

TEST ARTICLE: NTO | TREATMENT TIME: 18 Hours | SITEK STUDY NO.: 0985-3110

SPONSOR: US Army CHPPM | HARVEST TIME: 18 Hours | TRIAL NO.: B2

SOLVENT: DMSO | METABOLIC ACTIVATION: Yes ( ) No (X)

TREATMENT AND CONC. (µg/mL)	CELLS Scored	NUMBER AND TYPE OF ABERRATIONS																	NO. OF ABS. PER CELL	% CELLS WITH ABS.	P-VALUE IN CHI-SQUARE**
		NOT COMPUTED				Chromatid Type							Chromosme Type				Others				
						Simple		Complex					Simple	Complex							
		tg	sg	% pp	%e	tb	isb	tr	qr	cr	id	ci	sb	d	r	dm	pu	sd*			
Untreated A	100			0	0														0.00	0.0	
Untreated B	100			11	0														0.00	0.0	
Untreated A+B	200	0	0	5.5	0.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000	0.0	
Solvent A	100			0	0														0.00	0.0	
Solvent B	100			8	0														0.00	0.0	
Solvent A+B	200	0	0	4.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000	0.0	
1000 A	100			0	0														0.00	0.0	
1000 B	100			5	1														0.00	0.0	
1000 A+B	200	0	0	2.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000	0.0	= Solvent
2500 A	100			0	0														0.00	0.0	
2500 B	100			4	0	1													0.01	1.0	
2500 A+B	200	0	0	2.0	0.0	1	0	0	0	0	0	0	0	0	0	0	0	0	0.005	0.5	P=0.8947
5000 A	100			0	0														0.00	0.0	
5000 B	100			4	0														0.00	0.0	
5000 A+B	200	0	0	2.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000	0.0	= Solvent
MMC 0.4 A	100			0	0	12	11	8	3	15	6	4	9				1		0.69	46.0	
MMC 0.4 B	100			2	0	10		15	5		2	1	11		2				0.46	35.0	
MMC 0.4 A+B	200	0	0	1.0	0.0	22	11	23	8	15	8	5	20	0	2	0	1	0	0.575	40.5	P=0.0000

* sd = 10 aberrations in calculations.

**Statistical analyses done on the % cells with aberrations and with SITEK's historical data (0.39%) since concurrent solvent control was 0%.

In Chi-square test MMC compared to SITEK's historical untreated control data (0.13%) since solvent for MMC was water and concurrent value is 0%.

Verified by: QA VL SD JS

APPENDIX II

SITEK's HISTORICAL DATA FOR NEGATIVE CONTROLS (UNTREATED AND DMSO)

HISTORICAL DATA FOR NEGATIVE CONTROL (UNTREATED)
CHO IN VITRO CHROMOSOME ABERRATION ASSAY

NON - ACTIVATED SYSTEM

STUDY NUMBER	# OF METAPHASES SCORED	%CELLS WITH ABS.	Cells WITH ABS
0710-3110	200	0.0	0
0710-3110	200	0.5	1
0712-3110	200	0.0	0
0712-3110	200	0.5	1
0716-3110	200	0.0	0
0716-3110	200	0.5	1
0727-3110	200	0.0	0
0727-3110	200	0.0	0
0733-3110	200	0.0	0
0733-3110	200	0.0	0
0735/0736-3110	200	0.5	1
0735-3110	200	0.0	0
0736-3110	200	0.0	0
0736-3110	200	0.0	0
0738/0740/0741-3110	200	0.0	0
0738/0740/0741-3110	200	0.0	0
0739-3110	200	0.0	0
0739-3110	200	0.0	0
0745-3110	200	0.0	0
0745-3110	200	0.0	0
0760-3110	200	0.0	0
0760-3110	200	0.0	0
0761-3110	200	0.0	0
0761-3110	200	0.0	0
0771-3110	200	0.0	0
0771-3110	200	0.0	0
0788-3110	200	0.5	1
0790-3110	200	0.5	1
0790-3110	200	0.5	1
0795-3110	200	0.5	1
0795-3110	200	0.0	0
0799-3110	200	0.0	0
0800-3110	200	0.0	0
0800-3110	200	0.5	1
0820-3110	200	0.0	0
0833-3110	200	0.5	1
0833-3110	200	0.0	0
0835-3110	200	0.0	0
0836-3110	200	0.0	0
0836-3110	200	0.0	0
0840-3110	200	0.0	0
0840-3110	200	0.0	0
0849-3110	200	0.5	1
0849-3110	200	0.0	0
Total 44	8800		11

	MEAN	±	S.D.
RANGE : 0.0 - 0.5%	0.13	±	0.22
			n=44

OCT.2001 - SEP.2005

HISTORICAL DATA FOR NEGATIVE CONTROL (UNTREATED)
CHO IN VITRO CHROMOSOME ABERRATION ASSAY

ACTIVATED SYSTEM

STUDY NUMBER	PHASE	# OF METAPHASES SCORED	%CELLS WITH ABS.	CELLS WITH ABS
0710-3110	B1	200	1.0	2
0712-3110	B1	200	0.5	1
0716-3110	B1	200	0.5	1
0727-3110	B1	200	0.5	1
0733-3110	B1	200	0.5	1
0735/0736-3110	B1	200	0.0	0
0735-3110	B2	200	1.5	3
0736-3110	B2	200	1.5	3
0738/0740/0741-3110	B2	200	0.0	0
0739-3110	B2	200	0.0	0
0745-3110	B1	200	0.0	0
0760-3110	B2	200	0.0	0
0760-3110	B6	200	0.0	0
0761-3110	B2	200	0.0	0
0761-3110	B6	200	0.0	0
0771-3110	B1	200	0.0	0
0788-3110	B1	200	0.5	1
0790-3110	B1	200	0.5	1
0795-3110	B1	200	0.5	1
0795-3110	B2	200	0.5	1
0799-3110	B1	200	1.0	2
0799-3110	B2	200	0.0	0
0800-3110	B1	200	0.0	0
0820-3110	B1	200	0.0	0
0833-3110	B2	200	0.5	1
0836-3110	B1	200	0.0	0
0840-3110	B1	200	0.0	0
0849-3110	B1	200	0.0	0
Total 28		5600		19

	MEAN ±	S.D.
RANGE : 0.0 - 1.5%	0.34 ±	0.45
	n= 28	

OCT.2001 - SEP.2005

HISTORICAL DATA FOR DMSO
CHO IN VITRO CHROMOSOME ABERRATION ASSAY

NON-ACTIVATED SYSTEM

STUDY NUMBER	PHASE	# OF METAPHASES SCORED	%CELLS WITH ABS.
0710-3110	B2	200	0.5
0710-3110	B3	200	0.5
0716-3110	B1	200	0.0
0716-3110	B2	200	0.5
0733-3110	B1	200	0.0
0733-3110	B2	200	1.0
0760-3110	B1	200	0.5
0760-3110	B3	200	0.5
0761-3110	B1	200	0.5
0761-3110	B3	200	0.5
0790-3110	B1	200	0.5
0790-3110	B2	200	1.5
0799-3110	B1	200	0.5
0833-3110	B2	200	0.0
0833-3110	B3	200	0.0
0840-3110	B1	200	0.0
0840-3110	B2	200	0.0
0849-3110	B1	200	0.0
0849-3110	B2	200	0.5

RANGE : 0.0 - 1.5%

MEAN ± S.D.: 0.39 ± 0.39

n= 19

OCT 2001 - SEP 2005

HISTORICAL DATA FOR DMSO
CHO IN VITRO CHROMOSOME ABERRATION ASSAY

ACTIVATED SYSTEM

STUDY NUMBER	PHASE	# OF METAPHASES SCORED	%CELLS WITH ABS.
0710-3110	B1	200	1.5
0716-3110	B1	200	0.5
0733-3110	B1	200	0.5
0760-3110	B2	200	1.0
0760-3110	B6	200	0.0
0761-3110	B2	200	1.0
0761-3110	B6	200	0.0
0790-3110	B1	200	0.0
0799-3110	B1	200	0.0
0799-3110	B2	200	1.0
0833-3110	B2	200	0.0
0835-3110	B1	200	0.0
0840-3110	B1	200	0.5
0849-3110	B1	200	0.0

RANGE : 0.0 - 1.5%

MEAN ± S.D.: 0.43 ± 0.51

n= 14

OCT 2001 - SEP 2005

HISTORICAL %e AND % pp DATA FOR DMSO CHO IN VITRO CHROMOSOME ABERRATION ASSAY

NON-ACTIVATED

STUDY NUMBER	PHASE	# OF METAPHASES SCORED	% e	% pp
0710-3110	B2	100	0.0	0.0
0710-3110	B3	100	0.0	0.0
0716-3110	B1	100	0.0	0.0
0716-3110	B2	100	0.0	0.0
0733-3110	B1	100	0.5	0.0
0733-3110	B2	100	0.0	0.0
0760-3110	B1	100	0.0	0.0
0760-3110	B3	100	0.0	0.0
0761-3110	B1	100	0.0	0.0
0761-3110	B3	100	0.0	0.0
0790-3110	B1	100	0.0	0.0
0790-3110	B2	100	0.0	0.0
0799-3110	B1	100	0.0	0.0
0833-3110	B2	100	0.0	0.0
0833-3110	B3	100	0.0	0.5
0840-3110	B1	100	0.0	0.0
0840-3110	B2	100	0.0	0.5
0849-3110	B1	100	0.0	0.5
0849-3110	B2	100	0.0	0.5

%e RANGE : 0.0 - 0.5%
%pp RANGE : 0.0 - 0.5%

MEAN ± S.D.:

% e	0.03	±	**0.11**
%pp	0.11	±	**0.21**

***n* = 19**

OCT 2001 - SEP 2005

HISTORICAL %e AND % pp DATA FOR DMSO CHO IN VITRO CHROMOSOME ABERRATION ASSAY

SITEK Study No. 0985-3110

ACTIVATED

STUDY NUMBER	PHASE	# OF METAPHASES SCORED	% e	% pp
0710-3110	B1	100	2.5	0.0
0716-3110	B1	100	0.0	0.0
0733-3110	B1	100	1.0	0.0
0760-3110	B2	100	1.0	0.0
0760-3110	B6	100	0.5	0.0
0761-3110	B2	100	0.5	0.0
0761-3110	B6	100	0.5	0.0
0790-3110	B1	100	0.5	0.0
0799-3110	B1	100	0.5	0.0
0799-3110	B2	100	2.5	0.0
0833-3110	B2	100	0.5	0.0
0840-3110	B1	100	0.0	1.0
0849-3110	B1	100	1.0	0.0

%e RANGE : 0.0 - 2.5%
%pp RANGE : 0.0 - 1.0%

MEAN ± S.D.:

% e	0.85	±	**0.80**
%pp	0.08	±	**0.28**

***n* = 13**

OCT 2001 - SEP 2005

APPENDIX III

STUDY PROTOCOL AND PROTOCOL AMENDMENTS

TEST FOR CHEMICAL INDUCTION OF CHROMOSOME ABERRATIONS IN CULTURED CHINESE HAMSTER OVARY (CHO) CELLS WITH AND WITHOUT METABOLIC ACTIVATION

This protocol is presented in two parts. Part One is designed to collect specific information pertaining to the test article and study. Part Two describes the study design in detail. **Please complete all bolded sections in Part One and sign section 8.0 to approve the protocol.**

PART ONE

1.0 SPONSOR

1.1 Name: US Army Center for Health Promotion and Preventive Medicine
Aberdeen Proving Ground, MD

1.2 Address: Aberdeen Proving Ground, MD 21010

1.3 Sponsor's Study Coordinator: Gunda Reddy, Ph.D., DABT

2.0 TESTING FACILITY

2.1 Name: SITEK Research Laboratories

2.2 Address: 15235 Shady Grove Road, Suite 303
Rockville, Maryland 20850

2.3 Study Director: Jian Song, Ph.D.

3.0 STUDY NUMBERS

*3.1 Testing Facility's Study No.: 0985-3110

3.2 Sponsor's Study No.: Not Available

4.0 TEST ARTICLE

GLP's require that test article characterization information must be provided in the final report. This includes identification, lot number, purity, stability, source, and expiration date. As per regulatory requirements, lack of the above information will be cited as a GLP violation in the "Study Director's Compliance Statement" section of the final report.

*To be completed by the Testing Facility.

4.1 **Identification**

Name: 3-Nitro-1,2,4-Triazol-5-one (NTO)

Batch/Lot No.: BAE 07B 305-001

4.2 **Description**

Color: White

Physical Form: Powder

4.3 **Analysis**

Purity Information: 99.6%

Does the Sponsor require the use of a correction factor to account for impurity?

____ Yes __X__ No

If yes, what is the correction factor? _____

Determination of the test article characteristics as defined by Good Laboratory Practices will be the responsibility of the Sponsor. The specific GLP references for U.S. agencies are: FDA = 21 CFR, 58.105; EPA TSCA = 40 CFR, 792.105 and EPA FIFRA = 40 CFR 160.105.

4.4 **Stability**

Storage Conditions (check one):

____ Room Temperature __X__ Refrigerated (1-5°C)

____ Frozen (-10 to -20°C)

____ Other (please specify): _____

Expiration Date: Not Available

4.5 **Preferred Solvent (check one):**

__X__ H_2O ____ Culture Medium ____ DMSO ____ Acetone ____ Ethanol

____ Other (please specify): _____

____ To be decided by the Testing Facility

4.6 Special Handling Instructions:

Use Standard Laboratory Safety Practices For Avoiding Exposure To Hazardous Substances

5.0 REGULATORY AGENCY SUBMISSION

5.1 Test Design Specifications

This study protocol is designed to meet or exceed the U.S. EPA, ICH and OECD Guidelines specified in the following documents (1, 2, 3):

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Part 798, Health Effects Testing Guidelines, Subpart F, Section 798.5375, *In Vitro* mammalian cytogenetics. Revised July 1, 2002.

OECD Guideline for the Testing of Chemicals, No. 473. *In Vitro* Mammalian Chromosome Aberration Test. Adopted July 21, 1997.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genototxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996.

5.2 Good Laboratory Practices

This study will be conducted in compliance with the following Good Laboratory Practice standards:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 2005.

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 2005.

Japanese Ministry of Agriculture, Forestry and Fisheries, 11 NohSan, Notification No. 6283, October 1, 1999.

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984.

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45 [ENV/MC/ CHEM(98)17], Paris 1998.

Will this study be submitted to a regulatory agency?

_X_ Yes ____ No

If so, which agency(ies)? Worldwide

6.0 DOSING SOLUTIONS

The Sponsor will be responsible for determining the strength and stability of the dosing solutions. The U.S. requirements for analysis of dosing solutions are specified in: FDA = 21 CFR, 58.113; EPA TSCA = 40 CFR, 792.113 and EPA FIFRA = 40 CFR 160.113, and OECD GLPs, Section 6.2.

Does the Sponsor want dosing solution analysis?

____ Yes _X_ No**

If yes, please complete the rest of this section.

If requested by the Sponsor, SITEK Research Laboratories will determine the strength and stability of the dosing solutions. The method of analysis may be provided by the Sponsor, or if requested by the Sponsor, SITEK Research Laboratories will develop the method of analysis.

Alternatively, the Sponsor will be responsible for determining the strength and stability of the dosing solutions.

Dosing solution analysis will be performed by:

____ SITEK Research Laboratories ____ Sponsor***

What dosing solutions will be analyzed? _____

** Additional charges will apply. See Special Services price schedule.

*****Please note: All work pertaining to this study that is performed outside of SITEK is the responsibility of SITEK's Study Director. Therefore, as required by the GLPs, all of the following must be forwarded to the Study Director:**

- **All subcontract and/or Sponsor Quality Assurance audit findings and comments.**
- **Any deviations and/or amendments, if applicable.**
- **An original or copy of the analysis report.**
- **Location (address) of where the raw data from the analysis will be archived by the Sponsor or Subcontractor.**

If the subcontract work is not performed under the GLPs, a statement by the Sponsor informing SITEK's Study Director of such must be provided.

From the Range Finding Test?

_____ Yes_____ No

From the Assay?

_____ Yes_____ No

Which concentration(s)? _____

What amount of each concentration? _____

At what temperature should the dosing solutions be stored?

_____ Room Temperature **_____ Frozen (-10 to -20°C)**

_____ Refrigerated (1-5°C)

At what temperature should the dosing solutions be shipped?

_____ Room Temperature **_____ On Wet Ice**

_____ On Dry Ice

7.0 STUDY DATES

*7.1 Proposed Experimental Start Date: August 28, 2008

Defined as the first date the test article is applied to the test system.

*7.2 Proposed Experimental Completion Date: October 10, 2008

Defined as the last date on which data are collected directly from the study.

*7.3 Proposed Draft Report Date: October 24, 2008

7.4 Final Report: The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter.

*To be completed by the Testing Facility.

8.0 PROTOCOL APPROVAL

* _____ 8-26-08
Study Director — Date

_____ 8-26-08
Sponsor's Study Coordinator — Date

* _____ 8-26-08
Quality Assurance Manager — Date

* _____ 8-26-08
Safety Officer — Date

*To be completed by the Testing Facility.

STUDY DESIGN

PART TWO

9.0 PURPOSE

The purpose of this study is to evaluate the test article for its potential to cause genetic damage as manifested by induced chromosome aberrations in cultured Chinese hamster ovary (CHO) cells.

10.0 JUSTIFICATION FOR SELECTION OF TEST SYSTEM

The CHO cells have been used extensively in the Chromosome Aberration Assay and have been demonstrated to be effective in detecting the clastogenic activity of chemicals from a wide range of chemical classes (4-7).

11.0 ABBREVIATIONS

CHO	-	Chinese Hamster Ovary
CP	-	Cyclophosphamide
DMSO	-	Dimethyl Sulfoxide
G-6-P	-	Glucose-6-phosphate
HEPES	-	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIFBS	-	Heat-Inactivated Fetal Bovine Serum
KCl	-	Potassium Chloride
MMC	-	Mitomycin-C
MI	-	Mitotic Index
NADP	-	Nicotinamide-adenine Dinucleotide Phosphate (Sodium Salt)
DPBS	-	Dulbecco's Phosphate Buffered Saline (with Ca^{++} and Mg^{++})
PBS	-	Phosphate Buffered Saline (without Ca^{++} and Mg^{++})
RMI	-	Relative Mitotic Index

Complete Culture Medium - McCoy's 5A medium supplemented with 10% HIFBS, 2mM L-glutamine, 50 units/mL of penicillin and 50 µg/mL of streptomycin

Antibiotic-Free Medium - McCoy's 5A medium supplemented with 10% HIFBS and 2mM L-glutamine

12.0 INDICATOR CELLS

12.1 Source

The clone CHO-W-B1 of the CHO cell line, used in this study, originated at Litton Bionetics and was obtained by SITEK through the Environmental Health Research and Testing Laboratories, Lexington, Kentucky, in 1988. The doubling time of this cell line is approximately 12 hours, and its modal chromosome number is 21. The karyotype analysis of the cell line is periodically performed and documented at SITEK Research Laboratories.

12.2 Culture Conditions

The stock cultures of CHO cells are routinely grown in T-75 cm^2 sterile, plastic tissue culture flasks in antibiotic-free medium. The test cultures are grown in T-25 cm^2 plastic tissue culture flasks in complete medium. The cultures are kept in a humidified incubator maintained at approximately 37°C in an atmosphere of approximately 5% CO_2 and 95% air.

The stock cultures are routinely subcultured before confluency using 0.05% trypsin for dissociating the cells.

12.3 Stock Cultures

The CHO cells were propagated in antibiotic-free medium to obtain a sufficient number of cells for freezing a large number of stock ampules. The cells were cryopreserved in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) and 8% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. Prior to using the stock cultures for the test, representative ampules will be tested for contaminating microorganisms, including mycoplasma and also for karyotype stability. Stock ampules free of contaminating organisms will be used to initiate the stock cultures for the test. The cell cultures obtained from the stock ampules will be maintained by subculturing for a maximum of 15 passages and used to initiate cultures for the assays.

13.0 ROUTE OF ADMINISTRATION OF TEST ARTICLE

The test article will be administered *in vitro* directly or through a solvent compatible with the test system. This is the only route of administration available in this test system.

14.0 TEST SYSTEM IDENTIFICATION

All test cultures will be labeled in indelible ink with the SITEK study number, the test article concentrations/controls, the activation system, code number for the concentrations, A or B/C or D designating tubes receiving the same treatment, date of harvest and any other information that is pertinent to the Assay. Slides will be labeled with the SITEK study number, the code numbers for the concentrations tested, followed by A or B/C or D for the same treatment conditions and the date the slides are prepared.

15.0 CONTROL SUBSTANCES

15.1 Positive Controls

Mitomycin-C (MMC), which causes chromosome aberrations without metabolic activation, will be dissolved in water and used at 0.4 and/or 0.8 µg/mL for 3-hour treatment and 0.2 and 0.4 µg/mL for the 18-hour treatment in the non-activated system.

Cyclophosphamide (CP), which requires metabolic activation, will be dissolved in water and used at 7.5 and/or 12.5 µg/mL in the activated system.

The specific source, lot number, CAS No., storage conditions and expiration date of positive controls will be documented in the report.

If necessary, other appropriate positive controls can be used with the approval of the Sponsor.

15.2 Solvent Controls

The solvents used for dissolving the test article and positive controls will be used as the solvent controls. Culture medium, deionized, distilled water, DMSO (CAS #67-68-5), ethanol (CAS #64-17-5), and acetone (CAS #67-64-1) are some of the solvents which are compatible with this test system. If there is a need to use other solvents, the approval of the Sponsor will be obtained prior to their use.

The source, lot number and storage conditions of solvent controls will be documented in the report.

16.0 DOCUMENTATION

Detailed documentation of the procedures, results, and methods used for the analysis of the results of this study will be entered in a study notebook. The study notebook also includes copies of the protocol, protocol amendments and deviations, study reports, and all relevant communications with the Sponsor.

17.0 EXPERIMENTAL PROCEDURE

17.1 Determination of Solubility/Miscibility

In order to determine the appropriate vehicle for delivering the test article to the test system, or to determine the maximum achievable concentration in the solvent requested by the Sponsor, a solubility/miscibility test will be performed.

The test article will be tested for its solubility/miscibility in deionized, distilled water, DMSO, acetone, ethanol and/or other appropriate solvents. Solid and viscous liquid test articles will be tested for solubility in weight per volume, and nonviscous liquids will be tested for miscibility in volume or weight per volume. The solubility/miscibility test will be performed as described below.

For solid and viscous liquid test articles, the solubility test will consist of weighing out 25-100 mg aliquots of test article and adding solvent in 0.1 mL increments, with thorough mixing between additions, until the test article is dissolved or until 1.5 mL of solvent has been added to the vessel.

If the test article does not dissolve in 1.5 mL of solvent, more solvent will be added in aliquots of 0.5 mL until 5.0 mL has been added. The volume of solvent required for complete dissolution, and any additional observations, will be recorded in the study workbook. Test articles that do not dissolve in 5.0 mL of solvent will be recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

For nonviscous liquid test articles, a miscibility test will be conducted. 0.5 mL of each of the preferred solvents in 0.1 mL increments will be added to 0.5 mL aliquots of the test article. If the test article does not dissolve in 1.5 mL of solvent, more solvent will be added in 0.5 mL increments until 5.0 mL has been added. The resulting solution will be thoroughly mixed and observed for miscibility. The test article will be rated as either "not miscible," "partially miscible," or "completely miscible" in each of the preferred solvents. The miscibility rating and any additional observations will be recorded in the study workbook.

The solubility/miscibility test need not be performed if adequate information regarding the solvent and maximum soluble concentration is available.

The solubility or miscibility of the test article in culture medium will also be checked to determine the appropriate concentrations for the tests.

17.2 Preparation of Test Cultures

The CHO stock cultures grown in antibiotic-free medium and showing approximately 50-70% confluency will be harvested and used to prepare the test cultures for the Assay. The culture medium from the flasks will be discarded, and the cells will be washed with phosphate buffered saline (PBS). The cells will then be dissociated by trypsin at 37 ± 1.0°C, and resuspended in fresh complete culture medium. An aliquot of the cell suspension will be diluted to the appropriate concentration and counted using a cell counter. Based on the cell counts, a separate cell suspension in complete culture medium with $1x10^5$ cells/mL will be prepared to seed the test flasks. An appropriate number of T-25 cm^2 tissue culture flasks will be seeded with 5.0 mL of cell suspension to obtain test cultures with $5x10^5$ cells/flask. The cultures to be maintained beyond 48 hours after their initiation, will be seeded with an appropriately reduced number of cells (250,000-400,000 cells per flask) in order to avoid overgrowth of the monolayer. In the case of test articles, which react with plastic, 60 mL sterile glass culture flasks will be used instead of T-25 cm^2 plastic culture flasks. The flasks will be incubated for approximately 20-24 hours before treatment.

17.3 Preparation of Metabolic Activation System

The metabolic activation mixture will consist of phenobarbital/ß-naphthoflavone induced rat liver homogenate (S-9 fraction) (8) and the cofactor pool. The S-9 fraction will be stored at or below -70°C in small aliquots. The S-9 will be validated for acceptable levels of protein content and metabolic activity. Immediately prior to use, the S-9 will be thawed at room temperature and mixed with the cofactor pool to form the metabolic activation mixture which will consist of 4mM NADP, 5mM glucose-6-phosphate, 30mM KCl, 10mM $MgCl_2$, 50mM sodium phosphate (pH 7.4) and 100 µL/mL of S-9 fraction. This mixture will be diluted 1:4 by volume with serum-free medium and used in refeeding the cultures.

17.4 Preparation of Test Article

The desired amount of the test article will be weighed or measured as specified in the dilution

scheme which will be prepared prior to treatment for either the Range Finding Test or Assay. The stock solution of the highest concentration will be prepared by adding the appropriate volume of solvent to the test article just prior to use and thoroughly mixing the resulting solution until the desired dissolution is achieved. The remaining stock solutions specified in the dilution scheme will be prepared by a subsequent dilution or by dissolving the required amount of test article in the solvent at each concentration. When preparing the top dosing stock and any sub-sequent dosing stock with a viscous or non-viscous liquid, the test article should never be diluted more than 10-fold. In all treatments, the amount of solvent delivered to the target cultures will be limited to a level, which has no significant cytotoxic effect on the cells. If necessary, the test article may be added directly to the culture medium. If the test article is found to alter the pH of the culture medium to an extent that is toxic to the cells (9), either HEPES buffered medium will be used during treatment time or necessary adjustments will be made to the stock solution(s) or treatment medium prior to chemical exposure. A record of the pH measurements will be maintained in such cases.

17.5 Range Finding Test

If sufficient information is not available regarding the toxicity of the test article, a Range Finding Test will be performed in order to determine the test article concentrations that will produce 0-100% cytotoxicity. The test article will be weighed and a serial dilution will be prepared. If there is no solubility limitation, prior knowledge of cytotoxicity indicates differently, or the Sponsor specifies differently, the test article will be tested at eight to ten concentrations at a maximum concentration of 5000 µg/mL and lower concentrations covering four log dilutions. A solvent control will also be included in both the non-activated and activated systems. An untreated control (exposed only to water) will be included if a solvent other than water or culture medium is used. If a narrower concentration range or lower concentrations are required to determine the desired cytotoxic range, the Range Finding Test will be repeated.

The test cultures seeded approximately 20-24 hours earlier and are in the log phase will be used in the Range Finding Test. Duplicate cultures will be used at each concentration level.

In the non-activated system, the culture medium will be removed, and 5.0 mL of fresh complete medium will be added to each of the culture flasks. The cells will then be exposed to the test article for 3 hours. After the exposure period, the cells will be washed with DPBS, refed with complete medium, allowed to grow for 15 hours with 0.1 µg/mL Colcemid® present during the final 2 hours, and harvested 18 hours after the initiation of the treatment (1.5 x normal cell cycle time).

In the activated system, the medium will be removed and 5.0 mL of serum-free medium containing S-9 will be added to each of the culture flasks prior to treatment. The cells will be exposed to the test article for 3 hours by adding appropriate volumes of test article or dosing solutions to the culture medium. After the exposure period, the cells will be washed with DPBS, refed with complete culture medium, allowed to grow for 15 hours with 0.1 µg/mL Colcemid® present during the final 2 hours, and harvested 18 hours after the initiation of the treatment (1.5 x normal cell cycle time).

17.5.1 Determination of Relative Cell Growth (RCG) (2, 3)

After the Colcemid exposure, the medium with dividing cells in each flask will be transferred into labeled centrifuge tubes, the monolayer of cells will be washed with PBS, dissociated with 0.05% trypsin and resuspended in the collected medium. An aliquot of this cell suspension will be counted using an electronic cell counter. The number of cells per flask will be calculated for each

concentration, and the Relative Cell Growth (RCG) will be calculated according to the following formula:

$$RCG = \frac{\text{No. Cells in Test Flask}}{\text{No. Cells in Solvent Flask}} \times 100$$

17.5.2 Determination of Relative Mitotic Index (RMI)

The remaining cell suspension will be processed to determine the Relative Mitotic Index (RMI) as described below:

The cells will be collected by centrifugation, swelled in hypotonic KCl (0.075M), and fixed in methanol:glacial acetic acid (3:1) fixative. The fixed cells will be kept at 1-5°C. The cells will then be collected by centrifugation, resuspended in a small volume of fresh fixative, and dropped onto microslides to prepare chromosome spreads. The slides will be air dried, stained in Giemsa stain, and mounted in Cytoseal using #1 cover glasses.

The slides will be scored for Mitotic Index (MI). A total of 1000 cells will be scored from each concentration (500 from each duplicate flask) and the number of dividing cells recorded. The MI for each concentration level will be calculated using the following formula:

$$MI = \frac{\text{No. of Dividing Cells from 1000 Cells}}{10}$$

The RMI will be calculated as shown below:

$$RMI = \frac{\text{Test Concentration MI}}{\text{Solvent Control MI}} \times 100$$

The cytotoxicity will be evaluated on the basis of the RCG and/or RMI. If possible, a concentration causing approximately 50% reduction in RCG and/or RMI will be selected as the highest test concentration for the Chromosome Aberration Assay. In addition, three or more lower concentrations will be included in the Assay. If no cytotoxicity is observed at the maximum concentration tested, the Chromosome Aberration Assay will be performed at four decreasing concentrations starting with the maximum soluble concentration or one or two concentrations with precipitate. The actual concentrations for the assay, once determined, will be added to the protocol in the form of an amendment.

17.6 Chromosome Aberration Assay

The Chromosome Aberration Assay will be performed with a single harvest at 1.5 x normal cell cycle time.

Parallel Toxicity will be determined by the RCG of treated cells in comparison with solvent control. The procedure is the same as in the Range Finding Test.

The test cultures will be prepared as described in Section 17.2. Duplicate cultures will be treated and used at each concentration in each system in the evaluation of induced chromosome aberrations, RCG and RMI.

The treatment procedures for the Chromosome Aberration Assay will be the same as in the Range Finding Test. The cells will be treated with four or more concentrations of the test article, two concentrations each of the two positive controls and the solvent control in both the activated and non-activated systems. Untreated controls (only exposed to water) will be included in the Assay if a solvent other than water or culture medium is used.

In the non-activated system, the cells will be treated in complete medium for three hours. After the exposure period, the medium will be removed, the cells will be washed with DPBS, refed with complete medium and incubated for 15 hours with 0.1 µg/mL Colcemid® present during the last 2 hours. The cells will be harvested 18 hours after the initiation of treatment (1.5 x normal cell cycle time).

In the activated system, the cells will be treated in serum-free, S-9 containing medium for 3 hours. The removal procedure and incubation and harvest times are the same as in the non-activated system described previously.

After the Colcemid exposure, the cell suspension will be processed to determine the RCG and RMI as described in the Range Finding Test, section 17.5.1 and 17.5.2.

The same slides will be used to score chromosome aberrations, and scored "blind" in order to avoid bias on the part of the scorer(s). A total of three test concentrations, if possible, the highest of which causes approximately 50% reduction in RCG and/or RMI, one positive control concentration, the solvent and untreated controls will be scored from the activated and non-activated systems. Whenever possible, 100 metaphases will be scored from each of the two duplicate flasks. Consequently, 200 metaphases will be scored for each concentration for chromosome aberrations. Only cells with 19-23 chromosomes will be scored, and the microscope coordinates of each cell with findings will be recorded. In addition, the number of endoreduplicated and polyploid cells in a total of 100 metaphases per culture will be scored and recorded.

The types of Chromosome Aberrations scored and the corresponding abbreviations used are given below (10, 11):

1. Chromatid-type Aberrations

Simple:

tg	-	Chromatid gap - an achromatic region occurring along the length of a chromatid in which there is no misalignment.
tb	-	Chromatid break - a discontinuity occurring along the length of either of the two chromatids, in which there is a misalignment.
isb	-	Isochromatid break - a discontinuity occurring in both the chromatids at the same locus showing complete rejoining or sister chromatid union at both the broken ends or incomplete rejoining, i.e., only at one of the two broken ends.

Complex:

qr	-	Quadriradial - chromatid interchanges between chromosomes leading to four-armed configurations. This could be asymmetrical with formation of

a dicentric and an acentric chromatid, if union is complete, or symmetrical where there is no formation of a dicentric and an acentric chromatid.

tr - Triradial - isochromatid-chromatid exchanges resulting in three-armed configurations and sometimes fragments. The latter should not be scored as an independent aberration. The triradial could be monocentric or dicentric.

id - Interstitial deletion - intra-arm intrachanges resulting in deletion of small fragments which, however, stay in association with the parent chromatid.

ci - Chromatid intrachange - exchanges occurring between arms of the same chromosome resulting in asymmetrical (rings) or symmetrical configurations.

cr - Complex interchanges - multiarmed configurations resulting from breakage and reunion of two or more chromosomes.

2. Chromosome-type Aberrations

Simple:

sg - Chromosome gap - an achromatic region occurring in both chromatids of the chromosome at the same locus with no misalignment.

sb - Chromosome break - a discontinuity at the same locus in both chromatids, giving one acentric fragment which may be misaligned and a shortened monocentric chromosome, and where there is no sister chromatid union.

Complex:

d - Dicentric - an asymmetrical exchange between two chromosomes resulting in a chromosome with two centromeres with or without an accompanying acentric fragment which should not be scored as a second aberration.

r - Ring - inter-arm intrachange happening within the chromosome, leading to formation of a centric ring with or without a chromosome fragment. The fragment should not be scored as a second aberration.

dm - Double minutes - intra-arm intrachanges leading to tight acentric paired rings.

3. Other Aberrations

pu - Pulverized chromosome or chromosomes - shattering of chromatid material resulting in several minute pieces. The identity of the chromosome is not decipherable. Considered as a single aberration.

sd - Severely damaged cell - cell with ten or more aberrations.

pp - Polyploid cells - metaphases with multiples or approximate multiples of the haploid set of chromosomes. Not scored for structural aberrations.

e - Endoreduplicated cells - metaphases with paired duplicated chromosomes or diplochromosomes. They are not scored for structural aberrations.

The chromosome aberration data from the score sheets will be consolidated on a Summary Table. The number of aberrations per cell and the percentage of cells with one or more aberrations will be calculated separately for each duplicate culture and then pooled for each concentration. Chromatid gaps and chromosome gaps will not be included in calculating the percentage of cells with aberrations and the number of aberrations per cell. Of the remaining aberrations, each aberration scored will be counted as one, except a severely damaged cell (sd) which will be considered equal to ten aberrations in calculating the number of aberrations per cell. Endoreduplicated and polyploid cells will be recorded separately in percentages.

17.7 Statistical Analysis

The data for the percentage of cells with aberrations for each concentration will be compared to the solvent control values using a Chi-square test. The results will be considered significant if $p \leq 0.05$.

If the solvent control value is 0%, the data will be analyzed using the historical solvent control values. Statistical analysis will not be performed if the test concentration value is equal to or less than the concurrent or historical solvent control.

If a positive response is indicated by the Chi-square test, the Cochran-Armitage test (trend test) will be performed for evidence of a dose-related response (12). The trend test will be considered positive if $p \leq 0.05$.

17.8 Confirmatory Chromosome Aberration Assay

A confirmatory assay will not be performed if the definitive assay is positive either with and/or without activation.

A confirmatory Chromosome Aberration Assay without activation will be performed if the results of the definitive assay without activation produced a negative response. A continuous treatment up to the harvest time of 1.5 x normal cell cycle time (18 hours) will be performed (13), and the harvest time will be approximately 18-hours after the initiation of treatment (1.5 x normal cell cycle time). Negative results for the definitive assay with activation may require a confirmation on a case by case basis (13).

The harvest, RCG and RMI determination, and chromosome aberration scoring procedures will be the same as in the definitive assay. Parameters, such as test concentrations, may be adjusted in the confirmatory assay.

A confirmatory chromosome aberration assay with and without activation will be performed, if the definitive assay produces an equivocal response.

17.9 Criteria for a Valid Assay

1. In the solvent control, the percentage of cells with aberrations should not exceed 4%.

2. At least 25% of the cells scored in the positive control should show one or more chromosome aberrations.

3. At least one of the test concentrations scored should show approximately 50% reduction in the RCG and/or RMI. This requirement should not be applied to test articles where no apparent toxicity could be achieved at the maximum soluble concentration or highest allowable concentration.

17.10 Evaluation of Test Results

17.10.1 Positive Response

1. The test article will be considered to have caused a positive response in this assay if the test article shows a positive dose-response trend and a statistically significant increase ($p \leq 0.05$) over that of the solvent controls in the percentage of cells with aberrations at one or more concentrations.

2. In the event there is no positive dose response trend, at least two consecutive test concentrations show a statistically significant increase ($p \leq 0.05$) over that of the solvent controls in the percentage of cells with aberrations.

17.10.2 Negative Response

The test article will be considered to have caused a negative response if none of the test concentrations shows a statistically significant increase in the percentage of cells with aberrations.

17.10.3 Equivocal Response

The test article will be considered to have caused an equivocal response if one of the test concentrations shows a statistically significant increase in the percentage of cells with aberrations without an accompanying positive dose-response trend.

17.10.4 Other Considerations

The above criteria will be used as guidelines in evaluating the test results. However, the Study Director may take other factors into consideration in evaluating the test results.

18.0 PROTOCOL AMENDMENTS AND DEVIATIONS

If changes in the approved protocol are necessary, such changes will be documented in the form of protocol amendments and protocol deviations. Protocol amendments will be generated when changes in the protocol are made prior to performing a study or part of a study affected by the changes. In such cases, a verbal agreement to make such changes will be made between the Study Director and the Sponsor. These changes and the reasons for them will be documented and attached to the protocol as an addendum. Protocol deviations will be generated when the procedures used to perform the study do not conform to the approved protocol. The Sponsor will

be informed of these deviations, and as soon as practical, such changes, along with their reasons or explanations, will be documented and kept in the study notebook.

19.0 REPORT OF RESULTS

19.1 Content

The results of the study will be submitted to the Sponsor in the form of a final report. A draft report will be submitted before the final report is issued. The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter. The report will include, but not be limited to, the following:

1. Name and address of the testing facility and the dates on which the study was initiated and completed, terminated or discontinued.

2. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

3. Methods used to analyze the data.

4. The test and control substances.

5. Description of the methods used to perform the study.

6. The name of the Study Director and the names of other technical personnel or other professionals who participated in performing the study.

7. A description of the transformations, calculations or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.

8. The signed and dated reports of the Study Director or other professionals involved in the study.

9. The location where the raw data and reports are to be stored.

10. A statement from the Quality Assurance Unit.

19.2 Changes and Corrections to the Final Report

All changes to the final report will be in the form of report amendments which will include the reason(s) for the change, and these amendments will be added to the final report as an addendum.

20.0 ARCHIVES

The raw data, protocol, documentation, electronic file containing the data tables, and final report of the study will be maintained in the SITEK Research Laboratories Archives, 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

21.0 REFERENCES

1. United States Environmental Protection Agency, Title 40 Code of Federal Regulations Part 798, Health Effects Testing Guidelines, Subpart F, Section 798. 5375, *In Vitro* mammalian cytogenetics. Revised July 1, 2002.

2. OECD Guideline for the Testing of Chemicals, No. 473. *In Vitro* Mammalian Chromosome Aberration Test. Adopted July 21, 1997.

3. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80): 18198-18202, 1996.

4. Evans, H.J. Cytological Methods for Detecting Chemical Mutagens, In: Chemical Mutagens, Principles and Methods for their Detection, Vol. 4, Hollaender, A. (ed) Plenum Press, New York and London, pp. 1-29 (1976).

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9. Scott, D., S.M. Galloway, R.R., Marshall, M. Ishidate, D. Brusick, Jr., J. Ashby and B.C. Myhr. Genotoxicity under Extreme Culture Conditions. A report from ICPEMC Task Group 9. Mutation Res., 257: 147-204, 1991.

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11. Savage, J. R. Classification and relationships of induced chromosomal structural changes. J. Med. Genetics, 13:103-122, 1976.

12. Margolin, B.H., et al. Statistical analysis for *in vitro* cytogenetic assays using Chinese hamster ovary cells. Environ. Mutagen., 8:183-204, 1986.

13. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. Recommended for Adoption at Step 4 of the ICH Process on 16 July 1997 by the ICH Steering Committee.

PROTOCOL AMENDMENT 1

Amendment No.:	1
Sponsor:	US Army Center for Health Promotion and Preventive Medicine Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0985-3110
Sponsor's Study No.:	N/A
Test Article I.D.:	3-Nitro-1,2,4-Triazol-5-one (NTO)
Protocol Title:	Test for Chemical Induction of Chromosome Aberrations in Cultured Chinese Hamster Ovary (CHO) Cells with and without Metabolic Activation
Amendment No. 1:	Protocol page 2, section 4.5: Preferred solvent was changed from H_2O to DMSO.
Reason for Amendment No. 1:	The test article was not sufficiently soluble in H_2O.

APPROVED:

Jian Song, Ph.D.
Study Director

8-28-08
Date

PROTOCOL AMENDMENT 2

Amendment No.:	2
Sponsor:	US Army Center for Health Promotion and Preventive Medicine Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0985-3110
Sponsor's Study No:	N/A
Test Article ID:	3-Nitro-1,2,4-Triazol-5-one (NTO)
Protocol Title:	Test for Chemical Induction of Chromosome Aberrations in Cultured Chinese Hamster Ovary (CHO) Cells with and without Metabolic Activation
Amendment No. 2:	Protocol page 12, Section 17.5.2: The actual concentrations for the Definitive Assay were 5, 100, 500, 1000 and 5000 µg/mL both without and with activation. The actual concentrations for the Confirmatory Assay were 100, 500, 1000, 2500 and 5000 µg/mL without activation.
Reason for Amendment No. 2:	Protocol page 12, Section 17.5.2: The actual concentrations for the assay, once determined, will be added to the protocol in the form of an amendment.

APPROVED:

Jian Song, Ph.D.
Study Director

10-30-08
Date

APPENDIX IV

S-9 BATCH INFORMATION

MOLTOX POST MITOCHONDRIAL SUPERNATANT (S-9)
PRODUCTION & QUALITY CONTROL CERTIFICATE

LOT NO.: 2059 **SPECIES:** Rat **PREPARATION DATE:** September 7, 2006
PART NO.: 11-105 **STRAIN:** Sprague Dawley **EXPIRATION DATE:** September 7, 2008
VOLUME: 1 & 2ml **SEX:** Male **BUFFER:** 0.154 M KCl
TISSUE: Liver **INDUCING AGENT(s):** Phenobarbital - 5,6-Benzoflavone

REFERENCE: Matsushima, et. al., In: In Vitro Metabolic Activation in Mutagenesis Testing (F.J. de Serres, Ed.), Elsevier, 1976. p. 85.

STORAGE: At or below –70°C

BIOCHEMISTRY:

- PROTEIN

32.1 mg/ml — Assayed according to the method of Lowry et al., JBC 193:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	249.8
PROD	2B1	45.2
BROD	2B1	41.8
MROD	1A2	64.1

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 41.7, 7.9, 92.6, & 11.3 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 24 - 48 h incubation at 35°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. *his*+ Revertants	
EtBr/ TA98	CPA/ TA1535
545.6	436

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* 129:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames (*Mutat. Res.*113:173, 1983).

	µl S9 per plate/number *his*+ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5 µg)	103	166	382	574	784	935
2-AA (2.5 µg)	102	255	1419	1394	935	410

MOLECULAR TOXICOLOGY, INC.
157 Industrial Park Dr.
Boone, NC 28604 (828) 264-9099

APPENDIX V

CERTIFICATE OF ANALYSIS

BAE SYSTEMS

Ordnance Systems Kingsport, Tennessee

Certificate of Analysis for: NTO Per ORDNANCE SYSTEMS SPECIFICATION

Certificate No.: BAE 1686
Customer PO No.: 2007003323 Customer Part No.: N/A

	Batch Number:	10NTO7-3
	Containers Shipped:	14
	Lot Number:	BAE07B305-001
Characteristics	**Specs**	
% Purity by HPLC	99.0 - 100.0	99.6
Acidity, % as Nitric	0.01 Max	0.00
Exotherm Onset, Deg. C	250 Min	265
% Moisture	0.05 Max	0.03
Impact,cm		52
Appearance, Crystalline Solid	White to Pale Yellow	Pale Yellow Crystalline
Workmanship	PASS	PASS

Total Drums: 14 **Total Pounds:** 800

Prepared by: Neal Roberts

Digitally signed by Neal Roberts
DN: cn=Neal Roberts, c=US, o=BAE Systems, ou=Quality Assurance, email=neal.j.roberts@baesystems.com
Date: 2008.08.04 16:48:08 -04'00'

Neal Roberts, Quality Manager
Wednesday, February 28, 2007

[redacted]

Destination Control:
These items are covered by the United States Munitions List (USML) and is therefore subject to control by the U.S. Department of State. Export requires an export license, or other approval, issued by the U.S. Department of State.